REMARKS

Applicants respectfully request withdrawal of the finality of the present office action. The Examiner has raised new grounds of rejection which were not necessitated by Applicant's amendment. Specifically, there are six new grounds of rejections while no rejection was maintained from the previous office action. Applicants are unfairly burdened because they have not been given an opportunity to adequately address all of the Examiner's new concerns. For example, the rejection of claims 66-69 and 72-80 for allegedly lacking enablement for all fusion peptides could have been raised by the Examiner in the previous office action, but was not. This rejection was not necessitated by the Applicants' amendment. Applicants respectfully request withdrawal of the finality of the office action so that Applicants have sufficient opportunity to respond to the Examiner's new rejections. In the alternative, it is respectfully requested that this application be reconsidered in view of the following remarks and that all of the pending claims be allowed.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 66-69 and 72-80 were rejected for allegedly being indefinite for defining a broad range together with a narrow range. The Examiner cites MPEP § 2173.05(c) to support this rejection. However, MPEP § 2173.05(c) specifically deals with numerical ranges, which are not present in the claims of the present application. In addition, with respect to the Examiner's comments concerning *Ex parte Wu*, the claims do not recite broad language followed by "such as." Claim 66 defines members of epithelial cell proliferation-modulating agents in Markush Group format. As discussed in MPEP § 2173.05(h), it is permissible for a compound to be embraced by more than one member of a Markush group. "For example, the Markush group, 'selected from the group consisting of amino, halogen, nitro, chloro and alkyl' should be acceptable even though 'halogen' is generic to 'chloro.'" MPEP § 2173.05(h). This situation is identical to the situation in the claims of the present application. Therefore, since the claims are written in Markush Group format, claims 66-69 and 72-80 are definite and clear.

Rejection Under 35 U.S.C., First Paragraph, Enablement

Claims 66-69 and 72-80 were rejected for allegedly lacking enablement. The Examiner concedes that a fusion polypeptide comprising a collagen binding domain and an epithelial cell

proliferation-modulating agent, wherein the agent is hemopoietic growth factors (HGF), nerve growth factor or erythropoietin (EPO) is fully enabled. Applicants presume insulin is enabled, but request clarification. Applicants point out that insulin is known to be a cell proliferation agent. The Examiner, however, states that enablement is allegedly lacking when the agent is neu, inhibin α, inhibin β, Mullerian inhibitory substance, wnt, hst/ks3, stem cell factor, leukemia inhibitory factor or a receptor. The claims have been amended to define epithelial cell proliferation-modulating agents selected from the group consisting of insulin, nerve growth factor (NGF), NGF receptor, epidermal growth factor (EGF) receptor, neu, inhibin α, inhibin β, Müllerian inhibitory substance, tumor necrosis factor (TNF)-receptor (type 1), TNF-receptor (type 2), wnt-2, and hepatocyte growth factor (HGF) receptor (c-met). Support for this amendment can be found in the specification at least at page 11, line 19, to page 13, line2.

The Examiner's main arguments seems to be that the agents listed above are not epithelial cell proliferation-modulation agents. The specification, at least at page 10, lines 14-16 defines epithelial cell proliferation-modulation agents as any agent that can promote or inhibit epithelial cell growth or differentiation. The specification, at least at page 10, lines 21 to 23, defines growth factor as including molecules that function as growth stimulators or growth inhibitors. The specification, at least at page 11, line 19 to page 12, line 21, lists specific exemplary molecules that the specification defines as growth factors, which includes all of the specific agents listed in the claims. Therefore, the specification defines epithelial cell-proliferation modulation agents as including growth factors and growth factor receptors.

The Court of Appeals for the Federal Circuit (CAFC) has described the legal standard for enablement under § 112, first paragraph, as whether one skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *Amgen v. Hoechst Marion Roussell* 314 F.3d 1313 (Fed. Cir. 2003) and *Genentech, Inc. v. Novo Nordisk A/S*, 108 F.3d at 1365 (Fed. Cir. 1997). It has been well established that a patent need not teach, and preferably omits, what is well known in the art. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, (Fed. Cir. 1986). At the time of filing the present application, the agents defined by the claims were known epithelial cell-proliferation modulation agents. For example, as demonstrated by Kurada and White, *Apoptosis* 4(4):239-43 (1999) ("Kurada") (abstract enclosed) it was known that epidermal

growth factor receptor promotes cell proliferation and differentiation. As described in Kollias et al., *Ann. Rheum. Dis.* 58(Suppl. I):I32-I39 (1999) ("Kollias"), a copy of which is enclosed), tumor necrosis factor (TNF) receptors are found in soluble forms in biological fluids and mediate cellular responses such as cellular proliferation and differentiation (see page I36-I37). Cohen et al., *J. Neurosci. Res.* 31(4):622-34 (1994) ("Cohen") (abstract enclosed) describes the role of neu in Schwann cell proliferation and differentiation. Matzuk et al., *Nature* 360(6402):313-9 (1992) ("Matzuk") (abstract enclosed) states that inhibin alpha and beta are growth factors that are members of the transforming growth factor-beta family that regulate cell proliferation. Behringer, *Curr. Top. Dev. Biol.* 29:171-89 (1994) ("Behringer") (abstract enclosed) describes the *in vivo* roles of Mullerian inhibiting substance (MIS), which is a hormone that mediates cell differentiation of the Mullerian ducts. Dale et al., *Cancer Res.* 56(19):4320-3 (1996) ("Dale") (abstract enclosed) states wnt-2 is a mitogenic agent (i.e., induces mitosis) of epithelial cells. With respect to the Examiner's comment regarding receptors, it is noted that the receptors defined in the claims can be used as competitive inhibitors to decrease cell proliferation. Since the role of the agents defined by the claims as cell-proliferation modulating agents was well known to those of skill in the art, it would not require undue experimentation for those of ordinary skill in the art to make and use the polypeptides, nucleic acids, vectors, cells and methods defined by the claims. Therefore, claims 66-69 and 72-80 are enabled and Applicants respectfully request withdrawal of this rejection.

Rejections Under 35 U.S.C. § 103(a)

Claims 72-79 were rejected for allegedly being obvious over U.S. Patent No. 6,995,898 to Hall et al. ("the '898 patent"), in view of Carlini et al., *Kidney International* 55:546-53 (1999) ("Carlini").

Claims 66, 68 and 69 were rejected for allegedly being obvious over U.S. Patent No. 6,387,663 to Hall et al., ("the'663 patent") in view of Carlini.

Carlini was cited for describing the effect of EPO on endothelial cell proliferation. The claims define agents that modulate epithelial not endothelial cells. Furthermore, the claims as amended do not encompass EPO and are therefore not obvious over the cited references. Applicants respectfully request withdrawal of this rejection.

Double Patenting

Claims 72-79 were rejected on the grounds of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-8 of the '898 patent in view of Carlini.

Claims 66, 68 and 69 were rejected on the grounds of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-3 of the '663 patent in view of Carlini.

As discussed above, the claims, as amended, define epithelial cell modulating agents and do not encompass EPO. Therefore, the claims are patentable over the claims in the '898 or '663 patents in view of Carlini. Applicants respectfully request withdrawal of this rejection.

Allowance of the claims in this application is earnestly solicited. In the event that a telephone conversation could expedite the prosecution of this application, the Examiner is requested to call the undersigned at (404) 942-2747.

The required fee for a petition for a one month extension of time is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. However, please apply any charges or credits to deposit account 06 1050.

Respectfully submitted,

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1: Apoptosis. 1999 Aug;4(4):239-43.

Epidermal growth factor receptor: its role in Drosophila eye differentiation and cell survival.

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The Drosophila epidermal growth factor receptor (EGFR), functioning through the Ras/Raf/MAPK pathway, promotes cell proliferation and differentiation. Recent work has demonstrated that EGFR functions via the same Ras/Raf/MAPK pathway to promote cell survival. This review summarizes the role of EGFR in differentiation and survival during Drosophila eye development.

PMID: 14634274 [PubMed]

Ann Rheum Dis 1999;58:(Suppl I) I32-I39

The function of tumour necrosis factor and receptors in models of multi-organ inflammation, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease

George Kollias, Eleni Douni, George Kassiotis, Dimitris Kontoyiannis

Abstract

There is now good evidence to demonstrate that aberrations in tumour necrosis factor (TNF) production in vivo may be either pathogenic or protective and several plausible mechanisms may explain these contrasting activities. According to the classic pro-inflammatory scenario, failure to regulate the production of TNF at a site of immunological injury may lead to chronic activation of innate immune cells and to chronic inflammatory responses, which may consequently lead to organ specific inflammatory pathology and tissue damage. However, more cryptic functions of this molecule may be considered to play a significant part in the development of TNF mediated pathologies. Direct interference of TNF with the differentiation, proliferation or death of specific pathogenic cell targets may be an alternative mechanism for disease initiation or progression. In addition to these activities, there is now considerable evidence to suggest that TNF may also directly promote or down regulate the adaptive immune response. A more complete understanding of the temporal and spatial context of TNF/TNF receptor (TNF-R) function and of the molecular and cellular pathways leading to the development of TNF/TNF-R mediated pathologies is necessary to fully comprehend relevant mechanisms of disease induction and progression in humans. In this paper, the potential pathogenic mechanisms exerted by TNF and receptors in models of multi-organ inflammation, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease are discussed. Elucidating the nature and level of contribution of these mechanisms in chronic inflammation and autoimmunity may lead to better regulatory and therapeutic applications.

(*Ann Rheum Dis* 1999;58:(Suppl I) I32-I39)

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Tumour necrosis factor (TNF) is produced in response to infection or immunological injury and effects multiple responses, which extend beyond its well characterised proinflammatory properties[1] to include diverse signals for cellular differentiation, proliferation and death.[2] Part of the complexity of TNF mediated responses may be related to the apparent differential bioactivities of its soluble and transmembrane form[3] and the differential functioning of its two tumour necrosis factor receptors (TNF-Rs).[2] TNF has been for many years at the apex of factors showing dominant contribution to disease pathogenesis, especially chronic inflammation and autoimmunity. Its in vitro activities are now well understood and the signals transduced by the two TNF-Rs have been sufficiently detailed both at the molecular and the cellular level. However, the specific role of TNF and receptors in disease pathogenesis remains still poorly defined. Data discussed in this review, point towards multiple in vivo activities for this molecule. Firstly, the potent innate inflammatory activities of TNF seem central to disease induction and progression, particularly when sustained TNF expression is provoked. Evidence for direct effects of TNF on non-immune stromal cell types, which are important for the function of a given tissue or organ, is also available, and may offer alternative mechanisms for pathogenic contribution. Lastly, more recent data indicating direct modulation by TNF of the adaptive immune response may also be taken into consideration, to explain the beneficial or at times detrimental role of TNF in spontaneous models of autoimmunity. It is evident that no unique scenario is available to explain the role of TNF in inflammatory or autoimmune pathology. Rather, a diverse range of activities is expected to function in each model case, and most probably also in human disease. In figure 1, we have summarised the factors affecting TNF/TNF-R function in disease. Obviously, the diverse in vivo functions of TNF may significantly depend on the duration, quantity and quality of TNF signals. In addition, genetic background, locality and timing of TNF expression may also modulate the function of this molecule and diversify the end result of the immune response, either to the benefit or distress of the host. Experimental modification of these parameters in transgenic and knockout animal models of TNF mediated disease has been revealing.

Role of TNF in models of rheumatoid arthritis (RA)

The majority of the joint inflammatory disorders, typified by the manifestations of RA, are characterised by the hyper-proliferation of synovial tissue and the infiltration of blood derived cells resulting in the progressive erosion of the cartilage and bone. Genetic sus-

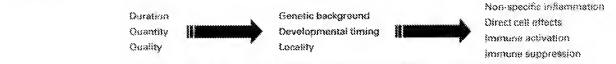

Figure 1 The diverse in vivo functions of TNF may significantly depend on the duration, quantity and quality of TNF signals. In addition, genetic background, locality and timing of TNF expression may also modulate the function of this molecule and diversify the end results of the immune response, either to the benefit or distress of the host.

ceptibility, physical stress, infectious agents and aberrant immune responses have all been implicated in the pathogenesis of RA.[4] The increased genetic linkage of specific HLA-DR haplotypes with RA[5] and the presence of experimental models of RA, induced in genetically susceptible animals after immunisation with collagen, led to the original assumption of autoimmune mechanisms playing a dominant part in both the induction and maintenance of the disease. However, the contribution of adaptive mechanisms in the pathogenesis of RA could not be confirmed at the clinical level, as both anti-CD4 and anti-CD5 clinical trials failed to induce a beneficial outcome.[6,7]

The role of lymphocytes in driving the arthritogenic response in the popular animal model of collagen induced arthritis (CIA) has also recently been challenged. Although T cell transfers and antibody depleting studies[8–10] had previously established the pathogenic potential of specific antigen reactive cells in this model, administration of collagen type II in CD4- and CD8-deficient mice resulted in the appearance (albeit with lower incidence) of the typical CIA profile, failing to provide evidence for an essential role in disease induction.[11] In view of the conflicting evidence on the role of an adaptive pathogenic immune response in CIA, we recently backcrossed Rag-1 deficient mice lacking mature lymphocytes into the CIA susceptible DBA/1 genetic background.[12] Collagen type II immunisation of these mice resulted in the development of arthritic lesions in their joints characterised by synovial hyperplasia with occasional inflammation as well as cartilage and bone destruction. The observed delayed onset and reduced severity of disease suggests that lymphocytes do play a significant, yet secondary part in disease induction. Evidence of synoviocyte proliferation in the absence of inflammation, after collagen administration in the DBA/1-immunodeficient mice, indicated an immediate responsiveness of this cell type to collagen. It may therefore be suggested that in susceptible genetic backgrounds, sequestration of collagen resulting from insults affecting joint integrity may activate the synoviocyte and cause pathology. These, non-antigenic properties of collagen may offer new clues as to the mechanisms operating in the pathogenesis of RA.

Early detection of a broad range of pro-inflammatory cytokines in RA biopsy specimens and explant cultures, established their importance in joint inflammation.[4] Among them, the significance of TNF in mediating the arthritogenic response, has been demonstrated by the amelioration of arthritic lesions in anti-TNF treated animal models of arthritis[13–15] and most importantly in human disease.[3] Perhaps, the most informative animal model, as to the TNF mediated mechanisms operating in arthritis, has been produced by the introduction of modified human TNF transgene in mice.[16] Based on the possible role of TNF 3'UTR on the translational repression of TNF mRNA, this region was replaced by that of the β-globin gene. The resulting transgenic mice developed a form of erosive arthritis with similar histological characteristics to RA. A similar form of arthritis appeared also in targeted mutant mice lacking the 3'AU-rich elements ($TNF^{\Delta ARE}$ mutant mice), confirming the role of these elements in the maintenance of a physiological TNF response in the joint.[16] In addition, neutralisation of arthritis by treatment of the hTNF transgenic mice with antibodies against TNF or the type I IL1 receptor established the idea that a functional hierarchy exists, in which the IL1R acts in series and downstream of TNF to effect TNF mediated arthritogenic responses.[17]

Absence of the 3'ARE elements from the mRNA of TNF results in chronic polyarthritis. A proposed mechanism that may affect the pathogenic outcome in arthritis, may be the inability of natural anti-inflammatory signals to suppress the destructive TNF expression in the arthritic joint. Anti-inflammatory mediators such as IL10, IL4 and TGF-β are abundant in rheumatoid joints, supporting the hypothesis of a counter-reaction compensating for the increased proinflammatory load.[18,19] However, this equilibrating response is apparently not sufficient to block disease progression. The inability of IL10 to efficiently suppress macrophage and/or synoviocyte TNF production because of the absence of the natural TNF 3'UTR (our unpublished observations), postulate a possible involvement of mutations affecting the expression of TNF (for example, functional mutations in TNF's ARE sequences) as aetiopathogenic factors in disease initiation or progression. This is further suggested by the fact that treatment of hTNF3'globin transgenic mice with cellular vectors producing IL4, IL10 and IL13 did not significantly affect development of arthritis in this model.[20] The minimal, if any, role of adaptive immunity in the development of arthritis in these models has been confirmed in studies demonstrating that the course of disease in hTNF transgenic and $TNF^{\Delta ARE}$ mutant mice is not affected by the absence of mature T and B cells.[16,21] This is in agreement with transplantation studies showing destruction of human cartilage by RA derived synoviocytes engrafted in the kidney capsule of SCID mice.[22,23]

Perhaps most importantly, the ARE deletion results in a profound spontaneous capacity of synoviocyte fibroblasts to produce TNF,[16,21]

Synoviocytes have been considered pivotal for the development of the arthritic reaction as they proliferate in response to TNF[36] and produce extracellular matrix-degradative enzymes and chemokines.[37 38] Their capacity to also produce TNF as a result of an ARE deletion, underscores their central role as both target and effector cells capable of initiating and maintaining the arthritogenic response. In an effort to assess if the TNF expressing synovial fibroblast is sufficient to induce disease, we have recently transferred clones of hTNF expressing synovial fibroblasts into the knee joint of histocompatible normal recipients. Migration of such fibroblasts from the injection site into other joints and different organs of the mouse could be demonstrated. Furthermore, four weeks after engraftment, a percentage of the mice developed polyarthritis with all the basic characteristics described for the donor (David Plows, Sylva Haralambous and George Kollias, unpublished observations). Interestingly, synoviocytes are seen to behave like tumour cells and can readily invade and destroy the environment that they home into.[37] In this context, the migration and homing of transferred TNF transgenic synoviocytes into multiple joints of recipient mice can be viewed as a metastatic process and the capacity to form a tumour mass may also rely on the angiogenic activities of TNF.

In conclusion, in models of TNF mediated arthritis, conceivably also in RA, the most critical phenomenon for both the initiation and perpetuation of disease seems to be the capacity of TNF to transform the nature of the synovial fibroblast, a cell with immense potential for unrestricted proliferation and autonomous invasion into the cartilage or bone. In this context, the contribution of non-specific inflammation and adaptive immunity seems to be in the regulation rather than initiation of disease. Understanding further the biology of the synovial fibroblast may offer new clues on additional molecules and mechanisms playing critical parts in the pathogenesis of arthritic disease.

Role of TNF in models of inflammatory bowel disease (IBD)

Pathogenesis of idiopathic IBD has been closely associated with the altered production of several pro-inflammatory cytokines. More specifically, the correlation between increased TNF production and IBD development has been exemplified in several animal models for this disease via the use of specific neutralising antibodies or cytokine gene knockouts.[39 40] Most convincing, the central role of TNF in the pathophysiology of human Crohn's disease (CD) has been overtly confirmed in clinical trials using a single dose of anti-TNF monoclonal antibody in patients with treatment refractory CD.[41] Furthermore, a similar to the human form of IBD appeared also in targeted mutant mice lacking the 3'AU-rich elements,[9] further substantiating the role of TNF in the development of diseases affecting the gut. The gut histopathological characteristics of the $TNF^{\Delta ARE}$ mice, including transmural inflammation, granuloma formation and ileal confinement, most closely resemble the human condition of CD[42] providing, for the first time positive evidence for TNF in inducing this form of IBD.[10]

TNF action in IBD has been considered mainly inflammatory through the activation of endothelial cells, induction of chemokines, and recruitment of neutrophils in the gut mucosa.[43] TNF has been shown to influence intestinal epithelial cell growth,[44] permeability,[45] and integrity via matrix metalloproteinase (MMP) production[46] and to induce epithelial cell apoptosis.[47] In addition, TNF has been implicated in the formation of bacterial induced granulomas through the induction of MCP-1 production by endothelial cells.[48] Intestinal epithelial damage is considered an early histopathological manifestation in CD[49] supporting the assumption that TNF plays a central part in initiating mucosal events in IBD. However, the action of TNF in the $TNF^{\Delta ARE}$ model of CD does not seem to be solely of an innate pro-inflammatory character. Intestinal pathology in these mice is heavily dependent on the presence of mediators or cells of the adaptive immune response, because in the absence of mature lymphocytes (for example, in backcrosses to Rag-1 deficient mice) intestinal inflammation is neutralised.[10] It is therefore possible that the chronic TNF production resulting from the ARE deletion shapes up a pathogenic lymphocytic response. TNF production has been numerously suggested to drive pathogenic Th1-like responses in concert with both IL12 and IFNγ.[40 50] Interestingly, activated mucosal T cells from CD patients demonstrate a characteristic Th1 cytokine profile associated with active disease.[42] Furthermore, a significant reduction of Th1-like lamina propria mononuclear cells has been reported in CD patients treated with the cA2 anti-TNF antibody, suggesting that anti-TNF treatment may proceed through the down regulation of mucosal Th1 cytokines.[49]

Our observation that in $TNF^{\Delta ARE}$ mice, IBD progression is also dependent on the function of the p75TNF-R, a receptor that is expressed mainly on hemopoietic cells, raises the intriguing possibility that TNF overproduction modulates the pathogenic response via the utilisation of this receptor. A suggested mechanism to explain disease pathogenesis in murine IBD has been provided by the CD4+ T cell transfer model of colitis.[50] In this model a T helper cell population ($CD4+CD45RB^{hi}$) isolated from healthy mice, elicits an aggressive from of colitis upon transfer to SCID mice. Anti-TNF/IFNγ treatment ameliorates disease progression indicating that this population elicits pathogenic Th1 responses. In addition, a counteracting T regulatory population ($CD4+CD45RB^{low}$) can suppress disease induction upon co-transfer. The presence of such regulatory T cell subsets in gut mucosa indicate a general homeostatic mechanism that should exist in this locality to counter balance the proinflammatory state resulting from the continuous bacterial assault from the gut lumen. Our data predict that the abnormal

TNF production results in the activation of the pathogenic T cell compartment, either through pro-inflammatory mechanisms or via direct suppression of the immunomodulatory compartment. It is even more appealing to predict that engagement of the p75TNF-R is differentially providing the suppressive signal for regulatory T cell populations. Mechanistically, chronic TNF production can induce a state of hypo-responsiveness via attenuation of TCR signalling[47] and/or induction of apoptosis.[48 49]

It remains unclear if localised or systemic events are necessary for the IBD phenotype to develop in the TNF$^{\Delta ARE}$ mice. In many animal models of IBD, a central immunological imbalance, for example, deficiency in IL2, TCR$\alpha\beta$, or overexpression of CD3, the profound alterations in T cell compartments favour the development of the disease.[50] It is therefore possible, that a central (thymic) deregulation will favour the development of the disease in TNF$^{\Delta ARE}$ mice. In light of what has been discussed so far, it is possible that TNF mediated dysregulation may impair the homeostatic interaction of these compartments leading to the expansion of pathogenic specificities. Further use of transgenic and mutant TNF mice as models of IBD, should allow better understanding of the intriguing activities of TNF in mucosal immunity.

Role of TNF in spontaneous and antigen induced models of multiple sclerosis (MS)

A pivotal role for TNF in the pathogenesis of inflammatory demyelinating disease of the central nervous system (CNS) has been suggested in several studies of MS in humans and in experimental autoimmune encephalomyelitis (EAE), an established autoimmune model for human MS. TNF is overproduced in the serum and cerebrospinal fluid of MS patients[51] and by resident and infiltrating cells[52] at sites of CNS injury. TNF can induce selective cytotoxicity of oligodendrocytes in vitro[53] and myelin damage in brain slices,[54] and is therefore directly implicated in the demyelinating process. The established activities of TNF in the initiation and maintenance of local inflammation, which are mediated by its known inductive effects on adhesion molecule expression[55] and macrophage activation,[56] may also contribute to the CNS lesions. In EAE, anti-TNF treatment completely prevents initiation of pathology and ameliorates the progression of established disease.[57 58] Moreover, similarly to the organ specific inflammatory phenotypes obtained in several TNF overproducing transgenic and mutant mice,[13 14 59 60] TNF overexpression in the CNS of transgenic mice has revealed the potential of this cytokine to induce an inflammatory CNS demyelinating disease.[61 62] In these transgenic models, TNF triggered CNS demyelinating disease is characterised by oligodendrocyte apoptosis, primary demyelination, and lymphocyte and macrophage infiltration of the CNS, resulting in loss of neural function.[63] Thorough characterisation of the demyelinating process at the histological level has validated TNF transgenic mice as accurate models for MS.[63] The mechanism of action of TNF in these models is not fully defined, but it could predictably involve recruitment and activation of macrophage/microglia, direct cytotoxicity of oligodendrocytes and/or triggering of a quiescent myelin reactive encephalitogenic component. However, removal of the mature lymphocytic population in these mice, by means of backcrossing to the immunodeficient Rag-1 knockout strain, did not change the development of primary demyelination demonstrating that TNF mediated pathology in this model does not require the adaptive arm of the immune response.[64] Similar mechanisms leading to primary demyelination with minimal, if any, immune involvement might also operate in MS.

The important role of TNF in inflammatory demyelination has also been examined in TNF deficient mice generated by gene targeting in embryonic stem (ES) cells. Myelin basic protein (MBP) induced EAE in TNF deficient mice crossed to the SJL/J strain was considerably delayed compared with an SJL.H-2b congenic control or the SJL/J strain itself,[65] in agreement with myelin oligodendrocyte glycoprotein (MOG) induced EAE in TNF or p55TNF-R deficient mice.[66 67] However, although TNF deficiency reproducibly delays the onset of EAE in different models of the disease, severe EAE with perivascular inflammation and primary demyelination eventually develops in TNF deficient mice,[65 66] indicating that other mediators may compensate for the absence of TNF during these processes. Although a more extensive quantitative comparison of the level of demyelination in wild type and TNF deficient mice is necessary, it is evident that TNF is not an obligatory mediator in the demyelinating process. The use of MBP as immunogen for EAE induction in TNF deficient mice also allowed an assessment of the role of TNF in autoimmune T cell development in general. The specificity of the T cell response of H-2b mice to MBP maps to a part of the molecule[68] that is expressed in the thymus of SJL mice[69] and the role of this thymic expression of MBP in T cell negative selection has recently been established.[71 72] In light of the ability of TNF to attenuate T cell receptor signalling,[47] a prediction has been put forward[73] that TNF expression imbalances in the thymus would have an effect on autoreactive T cell negative or positive selection. If TNF were influencing the strength of antigenic stimulation of thymocytes by a given autoantigen, it would possibly affect the susceptibility to the disease induced by this selecting autoantigen. However, analysis of the T cell responses of TNF deficient mice to MBP demonstrated that the avidity and the peripheral response of autoreactive T cells to MBP and the incidence and severity of MBP induced EAE in mice are not influenced by the TNF deficiency.[65] This result argues against an involvement of TNF in the generation and function of MBP specific self reactive T cells.

In conclusion, the important role of TNF in the development of inflammatory demyelination in the CNS has been revealed both in

spontaneous models of transgenic TNF overexpression in the CNS and in the antigen induced EAE model. In the former, TNF is clearly shown to cause primary demyelination. In EAE, TNF deficiency is shown to delay the onset of clinical disease. Studies in knockout mice have been useful in revealing the essential properties of disrupted genes as well as their redundant functions. For example, the finding that TNF deficiency does not completely prevent the development of demyelination during EAE underscores the existence of alternative pathways of demyelination. In that sense, the concept that there may be a single immunological pathway ultimately causing pathology in MS, is probably too simplistic. The presented studies indicate the existence of at least two distinct and seemingly independent immunological pathways leading to clinically indistinguishable types of myelin pathology classified in humans as "MS". Coexistence of multiple demyelinating pathways operating in concert could also explain the apparently unsuccessful trials of anti-TNF(LT)α[70, 71] or, anti-CD4 treatment[72] in established MS. Alternatively, mechanisms of disease induction as seen in the animal models, suggest that anti-TNF treatments in MS may be more efficacious at earlier stages where leucocyte trafficking and non-specific inflammation should play a most critical part in the amplification and perpetuation of disease. As additional information regarding initiating factors and common mediators of these pathways will emerge, better strategies for immune intervention will be discovered.

Role of the p75TNF-R in multi-organ inflammation

The two TNF receptors are known to mediate, either in cooperation or independently, a wide spectrum of cellular responses ranging from proliferation and differentiation to cytotoxicity or apoptosis.[1 73] In addition, soluble forms of the two TNF-Rs (sTNF-R) have been identified in biological fluids and are thought to have regulatory functions by affecting systemic TNF bioavailability.[74] Although knowledge of the biochemistry of TNF-R signal transduction is quite advanced,[74] understanding of the in vivo functions of the two TNF receptors remains vague. Using TNF or TNF-R knockout mice, the TNF/p55TNF-R pair was shown to have an essential role in many physiological processes including lymphoid organ architecture,[80 81] activation induced T cell death,[82] and resistance against bacterial,[80 82 83] parasitic,[84] or viral infections.[85] A dominant role of the p55TNF-R has also been apparent in at least the induction phase of several TNF mediated pathologies, including endotoxemic shock in the presence of TNF sensitising agents,[82 83] or in several transgenic models of disease where deregulated production of TNF has been pathogenic.[15 29 86] In contrast, using p75TNF-R knockout mice, there has been very little evidence for a specific involvement of the p75TNF-R in physiology or experimental models of disease.[1 76] This failure to demonstrate an in vivo independent activity of the p75TNF-R in the knockout system, together with ample in vitro evidence for a cooperative role of this receptor in p55TNF-R mediated responses, have led to the concept that the p75TNF-R serves an accessory role in increasing p55TNF-R signalling.[87] It should be noted, however, that some in vivo functions of this receptor have been recently revealed in murine models of cerebral malaria,[88] in concanavalin-A induced hepatitis[89] and in the allergen induced migration of Langerhan's cells.[90] Interestingly, so far, the in vitro activities of the p75TNF-R have always been associated with the capacity of this molecule to induce the proliferation of thymocytes and peripheral T cells,[91 92] but also the death of activated CD8+ T cells.[93 95] Indeed, in contrast with the p55TNF-R, which is expressed on almost every cell type, the expression patterns of the p75TNF-R are restricted to endothelial cells and cells of haemopoietic origin,[96 97] suggesting that the in vivo activities of this molecule may only be evident in phenomena involving these specific cell types. In addition, the well documented preference of the p75TNF-R to signal upon binding to transmembrane,[3] rather than soluble TNF,[98] may hamper attempts to identify an in vivo role for this receptor in models where soluble TNF overexpression or p55TNF-R dependent activities often dominate the ensuing phenotypes.

The independent in vivo activities of the p75TNF-R, have recently been investigated in transgenic mice that express constitutively increased, yet disease relevant levels of a wild type human p75TNF-R.[97] These transgenic mice carry the entire genomic region of the human p75TNF-R gene, and expression analysis has revealed that tissue patterns of transgene expression were comparable to those seen for the endogenous murine p75TNF-R. Furthermore, fold induction in the levels of wild type versus transgenic soluble human p75TNF-R, correlated with increases in the levels of the sp75TNF-R between healthy and diseased human sera. It is important to note that heterozygous transgenic mice produce constitutively, threefold to fourfold higher levels of soluble hup75TNF-R in comparison with controls, develop a chronic perivascular inflammatory pathology in the liver, pancreas and lung at 2–3 months of age. Moreover, homozygous mice producing constitutively soluble hup75TNF-Rs at levels similar to those seen for the endogenous murine p75TNF-R in LPS treated normal mice (that is, about 200 ng/ml), develop a severe pathology characterised by multi-organ inflammatory lesions, and liver and pancreas necrosis, that lead to their premature death between 2 and 4 weeks of age.[97] These results indicate that the severity of the developing inflammatory phenotypes correlates positively with the levels of soluble human p75TNF-R measured in diseased sera. Interestingly, serum sp75TNF-R levels measured in several human inflammatory diseases, including AIDS, are usually threefold to fourfold increased over normal controls,[99] while septic shock patients display an approximately fivefold increase.[83] In addition, these levels strongly correlate with the clinical stage

and the progression of disease and can be of predictive value.[100]

With reference to human disease, a plethora of studies have indicated that chronic increased production of the soluble p75TNF-R demarcates fatal human inflammatory conditions including sepsis,[99] chronic viral hepatic diseases,[101] acute respiratory distress syndrome,[102] acute pancreatitis,[103] lupus,[104] rheumatoid arthritis[105] and AIDS.[98] The actual involvement of soluble TNF receptors in disease pathogenesis remains controversial and it has been suggested that they may act both as antagonists of TNF action by competing with its cell surface receptors but also as agonists by protecting TNF from degradation and therefore stabilising its activity.[78] In addition, shedding of both TNF receptors occurs in both a constitutive and inducible manner and is thought to serve in rendering cells temporarily unresponsive to TNF.[78] It is conceivable, however, that sustained upregulation of the p75TNF-R may lead to both, an increase of shed soluble receptors levels, but also to a chronic accumulation of the receptor on the cell surface. We have confirmed this hypothesis both in the transgenic system of p75TNF-R overexpression,[97] but most importantly also in a group of septic patients, where we have recently detected a parallel upregulation of the soluble p75TNF-R in their sera and of the membrane bound form of this receptor on their peripheral blood mononuclear cells. It is therefore evident that increased levels of shed p75TNF-Rs, as reported in human disease, reflect a similar upregulation of the membrane bound form of the receptor that may consequently interfere with immune homeostasis and disease pathogenesis. Importantly, also, sustained upregulation of the p75TNF-R during human disease may not be accompanied by chronically increased levels of TNF. Indeed, in a recent study investigating kinetics of soluble TNF-R production after leakage of high doses of TNF in the circulation of patients undergoing isolated limp perfusion treatment, it has been observed that levels of soluble p75TNF-R remain high, long after TNF disappears from the circulation, indicating regulatory and perhaps also functional disengagement from TNF.[106]

Interestingly, the lethal inflammatory phenotype developing in the hup75TNF-R transgenic mice is shown to evolve independently of the presence of TNF, LTα, or the p55TNF-R.[97] This finding suggests a physiologically significant role for ligand independent signalling of the p75TNF-R, a receptor known to be strongly induced during the course of an inflammatory response.[107] There is substantial evidence in vitro, that induced production of members of the TNF-R family, such as the p75[108 109] or the p55TNF-R,[110] Fas,[110] CD40,[108 111] or the p75NGF-R[112] may lead to spontaneous signalling even in the absence of ligand. The in vivo relevance of this phenomenon, especially for the p75TNF-R, may be of pivotal importance in many clinical conditions including sepsis. Notably, although the TNF/p55TNF-R system seems to operate only in an initial narrow window of time during clinical sepsis,[113] soluble p75TNF-R levels are found constantly increased, correlate with sepsis scores and show maximal values in patients that do not survive.[99] After the disappointing outcome of anti-TNF trials in sepsis, it is tempting to speculate that the increased mortality seen specifically in patients treated with a soluble p75TNF-R-IgG protein,[114] may have been attributable to an agonistic effect of this specific construct on the endogenous cell surface p75TNF-R either by interference with the shedding of this receptor or by mounting an agonistic humoral immune response. Taking into account the observed adverse effects of enhanced p75TNF-R production in transgenic mice, it is conceivable that strategies aiming at inhibiting induced self association or constitutive signalling of this receptor may offer new opportunities of interfering therapeutically with its putative harmful involvement in disease pathogenesis.

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1: Nature. 1992 Nov 26;360(6402):313-9.

Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice.

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The inhibins are alpha:beta heterodimeric growth factors that are members of the transforming growth factor-beta family. To understand the physiological roles of the inhibins in mammalian development and reproduction, a targeted deletion of the alpha-inhibin gene was generated by homologous recombination in mouse embryonic stem cells. Mice homozygous for the null allele (inhibin-deficient) initially develop normally but every mouse ultimately develops mixed or incompletely differentiated gonadal stromal tumours either unilaterally or bilaterally. Inhibin is thus a critical negative regulator of gonadal stromal cell proliferation and the first secreted protein identified to have tumour-suppressor activity.

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1: J Neurosci Res. 1992 Apr;31(4):622-34.

Expression of the neu proto-oncogene by Schwann cells during peripheral nerve development and Wallerian degeneration.

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The neu gene, which encodes a putative tyrosine kinase growth factor receptor termed p185neu, was originally identified as a dominant transforming gene in neurogliomas and schwannomas induced by transplacental treatment of rat embryos with ethylnitrosourea. The present studies were undertaken to determine the expression pattern of the neu gene in peripheral nerve. Northern blot analysis of total RNA isolated from rat sciatic nerves demonstrated prominent neu mRNA expression on postnatal days 1 and 7, with substantially lower expression up to adulthood. Immunohistochemical studies confirmed expression of p185neu by Schwann cells (SC) in developing sciatic nerve and minimal p185neu immunoreactivity in adult nerves. However, neu mRNA and p185neu protein progressively increased following sciatic nerve transection in adult animals. In addition, neu mRNA and p185neu were found in neonatal rat sciatic nerve SC and several SC-derived cell lines. In resting SC, neu mRNA was expressed at a low level, but was greatly increased by treatment with forskolin and glial growth factor. These studies demonstrate that the neu gene and its protein product, p185neu, are expressed by SC both in vivo and in vitro and suggest that p185neu plays a role in the regulation of SC proliferation or differentiation.

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Links

The in vivo roles of mullerian-inhibiting substance.

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The fetal testis functions as the sex differentiator by imposing a masculine pattern of development upon a genetic program that is inherently female. Two hormones produced by the fetal testis mediate the differentiation of the mullerian and Wolffian ducts (Figs. 1 and 4). MIS actively inhibits the development of the mullerian ducts, and testosterone induces the differentiation of the Wolffian ducts. The absence of these two hormones during fetal development in the female (the hormonal equivalent of no testes) permits mullerian duct differentiation and does not induce Wolffian duct development. The in vivo outcomes of ectopic MIS exposure or MIS deficiency illustrate the balance required to coordinately differentiate and cause regression of the respective male and female genital ducts. The observations made in the MIS-deficient mice demonstrate that codevelopment of both genital duct systems interferes with normal development of both systems and ultimately interferes with reproduction and fertility. Thus, reproduction and fertility in mammals appear to be most efficient if only one type of genital duct system develops. The phenotypes of the MIS-overexpresing transgenic mice and the MIS-deficient mice are similar yet different. Some of the explanations that might reconcile these differences probably lie with the receptor for MIS. Since the MIS-overexpressing transgenic mice are exposed to pharmacological levels of MIS during development, it seems possible that this may lead to productive interactions with other related receptors. Candidate genes have been isolated for the MIS receptor that are membrane-bound serine/threonine kinases (Baarends et al., 1994; di Clemente et al., 1994) similar to those cloned for the TGF-beta (Lin et al., 1992) and activin (Mathews and Vale, 1991) type II receptors. Interestingly, expression of these putative MIS receptor genes is localized by in situ hybridization to the mesenchymal cells adjacent to the mullerian ducts, suggesting that MIS most likely alters the surrounding mesenchyme to elicit mullerian duct regression. Experiments are underway to isolate the mouse MIS receptor gene to thereby generate MIS receptor-deficient mice and to compare the phenotype with the MIS gain-of-function and loss-of-function animals. Isolation of the human MIS receptor gene

will facilitate the identification of human PMDS patients with normal levels of MIS that have mutations in the MIS receptor gene. Finally, studies of the MIS receptor gene will open up avenues for the molecular characterization of signal transduction pathways that mediate mullerian duct regression and Leydig cell proliferation control.

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1: Cancer Res. 1996 Oct 1;56(19):4320-3.

Compartment switching of WNT-2 expression in human breast tumors.

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WNT-2 is a secreted polypeptide with mitogenic effects in murine mammary epithelial cells, but its role in human cancer is unknown. Using RNase protection analysis of primary cell preparations and in situ hybridization analysis, we report that WNT-2 is expressed at low levels in normal human breast fibroblasts but not in epithelial cells. WNT-2 was found to be expressed at high levels in both the epithelium and stroma of 5 of 11 infiltrating carcinomas and 2 of 6 fibroadenomas. The high level of WNT-2 expression in tumor epithelium suggests that tumorigenesis may involve the ectopic expression of WNT-2 and the creation of an autocrine Wnt signaling loop.

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